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We have studied effects of CEES alkylation upon the ability of purified laminin 5 to bind keratinocytes, and to bind type VII collagen NC1 domain in vitro. Alkylation of laminin 5 eliminates keratinocyte binding both when laminin 5 is treated with CEES as a solid growth substrate, and when laminin 5 is first alkylated in solution and then bound to the plastic growth surface. Alkylated laminin 5 retains its ability to bind to the type VII collagen NC1 domain. Alkylation of NC1 altered its solubility, and its binding to laminin 5 could not be evaluated.

Purified, untreated laminin 5 promoted the adhesion rate of keratinocyte sheet grafts to wounds in nude mice. Antibodies to laminin 5 that interfere with epithelial cell binding in vitro slowed the closure of wounds made in human skin grafted to nude mice. However, the addition of exogenous laminin 5 did not significantly increase the rate of closure of wounds made to wounds in the same system. Blisters made using CEES treatment in this system resulted in wounds too irregular to allow testing of possible effects of laminin 5 treatment upon closure of the wound.

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FOREWORD

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Introduction

The basement membrane changes due to sulfur mustard induced vesication mimics those of junctional epidermolysis bullosa (JEB) with regard to the plane of cleavage and the distribution of basement membrane structural proteins between the blister roof and floor. JEB results from a failure of epidermal-basement membrane attachment due to the absence or mutation of laminin 5, the absence of its hemidesmosomal receptor integrin $\alpha 6\beta 4$, or the absence of the hemidesmosomal protein collagen XVII. One interpretation of existing evidence suggests that alkylation of laminin 5 underlies initial blister formation, while generalized alkylation of laminin 5 and other basement membrane components may prevent their recognition by integrins involved in epithelial cell migration and result in slowed blister coverage. We have preliminary data that indicates that exogenous laminin 5 promotes keratinocyte migration and basement membrane assembly. We suggest that laminin 5 applied in solution to the blister floor and to the neoepithelium will speed epithelial coverage, provide temporary stabilization of epithelial attachment, and thereby promote the normal remodeling of the derivatized basement membrane. We will directly test this interpretation by:

(i) determining the effects of chloroethylethyl sulfide (CEES) alkylation of laminin 5 upon its ability to promote attachment of keratinocytes, and to bind collagen VII. To determine the effect of alkylation of collagen VII NC-1 domain on its ability to bind laminin 5.

(ii) determining the distribution of (CEES) alkylation products incorporated into laminin 5 and collagen VII following alkylation of organotypic skin models.

(iii) evaluating the utility of exogenously applied laminin 5 to promote keratinocyte coverage of epithelial erosions caused by treatment of full thickness fetal bovine skin grafts and full thickness human skin grafts to nude mice with CEES.

The studies are expected to proceed as follows:

Year 1 Goals:

Purification of laminin 5 and type VII collagen from keratinocyte culture medium by immunoaffinity chromatography. Alternately, type VII collagen NC-1 domain will be

purified from extracts of human amnion. 10-15 mg of each reagent will be required each year of the project. Continued production of both reagents will occur during years 2

Optimization of laminin 5 and type VII collagen alkylation. Determination of extent of alkylation of laminin 5 α3 G domain (integrin α6β4 binding site).

Optimization of grafting of human and fetal bovine skin to nude mice, blistering of grafts, and immunohistological procedures.

Year 2 Goals:

Alkylation of laminin 5 present in organotypic models.

Solid phase binding studies of laminin 5 with collagen type VII collagen, before and after alkylation.

Keratinocyte attachment studies to alkylated and non-alkylated laminin 5.

Preliminary treatment of vesicated grafts with laminin 5, and subsequent analysis. Determinization of laminin 5 concentration dependence.

Background

Summary. The cutaneous and mucosal basement membranes are composed of independent but associated networks of type IV collagens and laminins, into which are intercalated proteoglycans and fibronectin. While once it was believed that the major laminin present in these basement membranes was laminin 1 (EHS laminin), it is now clear that epithelial laminins are a genetically and functionally distinct laminin subclass comprised of laminins 5, 6 and 7 (α3β3γ2; α3β1γ1; and α3β2γ1 respectively). If present at all, laminin 1 is only a minor component. Small amounts of additional laminins (laminin 8 - α4β1γ1, and laminin 10 - α5β1γ1) are also present but their function is unknown. Recent evidence indicates that laminin 5 is essential to epithelial-basement membrane stability as it is the key bridging molecule between the keratinocyte hemidesmosomal integrin α6β4, and the anchoring fibril protein collagen VII. Laminin 5 specifically binds to both components. Mutations in any of the three subunits of laminin 5 that prevent its intracellular assembly result in lethal blistering characteristic of Herlitz junctional epidermolysis bullosa (JEB). Point mutations in critical subdomains of laminin 5, or the absence of integrin α6β4, or the absence of another hemidesmosomal transmembrane protein collagen XVII result in mild JEB.

The dermal-epidermal junction of skin contains novel ultrastructural elements along the basolateral epithelial surface and underlying basement membrane zone known as anchoring complexes (reviewed in Burgeson, 1996; Gerecke et al, 1994; Burgeson, 1993). Briefly, these

unique structures are comprised of *hemidesmosomes,* into which the cytokeratin filaments insert (Coulombe et al, 1991). The hemidesmosomes include the intracellular proteins hemidesmosomes-1 (Owaribe et al, 1990; Hieda et al, 1992) (a 500 kDa homologue of plectin); BPAG1 (Tanaka et al, 1991; Sawamura et al, 1991; Garrod, 1993) (a dimer of 230kDa desmoplakin homologues); and the transmembrane proteins BPAG2, (Diaz et al, 1990; Giudice et al, 1991; Hopkinson et al, 1992; Li et al, 1992) also known as collagen XVII (Li et al, 1993); and the integrin α6β4 (DeLuca et al, 1990; Carter et al, 1990; Sonnenberg et al, 1990; Kurpakus et al, 1991). Extending from the hemidesmosomes are fine filaments, the *anchoring filaments*, that appear to bridge the hemidesmosomes with the basement membrane lamina densa (Ellison and Garrod, 1984). The anchoring filaments are composed of the exodomains of α6β4 and collagen XVII which probably account for the outer dense plate of the hemidesmosome, and laminins 5, 6 and 7. Continuous with the anchoring filaments beneath the lamina densa are centrosymmetrically cross-striated fibers, the *anchoring fibrils*, composed primarily of collagen VII (Sakai et al, 1986). The anchoring fibrils associate end-to-end through structures known as anchoring plaques, thus forming an extended network of anchoring fibrils that physically entrap fibrous elements within the papillary dermis (Keene et al, 1987). The ultrastructure of the anchoring complexes suggest that they provide a continuous series of interacting proteins that link the epithelial cytokeratins with the dermal fibers, thereby stabilizing the epithelial attachment to the stromal matrix in order to resist external friction (Garrod, 1993).

The molecular details of the interactions that must occur within the anchoring complexes are only beginning to be described. The deduced primary structure of the hemidesmosomal proteins BPAG1(Sawamura et al, 1991), α6β4 (Tanaka et al, 1989; Hogervorst et al, 1990) and collagen XVII (Li et al, 1992) are known. However, the functions of included subdomains are only indirectly understood. α6β4 is an unusual integrin due to the extreme length of the β4 cytoplasmic domain (Hogervorst et al, 1990). It is suspected, although not formally proven, that this cytoplasmic tail interacts with hemidesmosomes1 and/or BPAG1 to secure their attachment, and therefore the keratin filament attachment to the plasma membrane. Likewise, it is assumed that collagen XVII also binds hemidesmosomes1 and/or BPAG1 serving a related purpose. The primary ligand of α6β4 is the α3 chain present in laminins 5-7 (Sonnenberg et al, 1993; Niessen et al, 1994). Steric blocking of this domain with antibodies directed against either α6 or β4 can prevent recognition of laminin α3 by α6β4 (Delwel et al, 1993; Niessen et al, 1994; Rousselle and Aumailley, 1994) and prevent hemidesmosome formation (Jones et al, 1991; Kurpakus et al, 1991; Langhofer et al, 1993). The ligand for the collagenous exodomain of collagen XVII, if one exists, is unknown.

The structure-function relationships of the anchoring filament proteins, laminins 5, 6 and 7 are partially understood (Figure 2). By rotary shadowing, laminin 5 appears as a dumbbell (Rousselle et al, 1991), and laminins 6 and 7 appear as "Y" shaped images (Champliaud et al, 1996) as opposed to the cruciform images obtained from laminins 1-4 (Engel, 1992). The shape of laminin 5 results from severe truncation of the short arms of the primary translation product (Kallunki et al, 1992; Vailly et al, 1993; Gerecke et al, 1994; Ryan et al, 1994) and due to proteolytic processing of the α3 and γ2 chains (Marinkovich et al, 1992). As a result, the final tissue form cannot self-polymerize, co-polymerize with other laminins to form a network (Yurchenco et al, 1992), nor can it bind nidogen (Mayer et al, 1995), and thereby associate with the collagen IV network or with perlecan. Yet laminin 5 does associate with the lamina densa as seen in RDEB cases in which collagen VII is lost and blisters form below the lamina densa, but the basement membrane remains associated with the displaced epithelium (Marinkovich, 1993; Uitto and Christiano, 1992; Uitto et al, 1992). In contrast, the loss of laminin 5 seen in Herlitz JEB causes separation within the lamina lucida, suggesting that laminin 5 is the primary tether between the epithelial cells and the basement membrane. Two mechanisms appear to facilitate the association of laminin 5 with the lamina densa. Laminin 5 covalently associates with either laminin 6 or laminin 7. The disulfide bond linking the complexes is likely to be between the VI domains of β3 of laminin 5 and the III domain of α3 in laminins 6 or 7 (Champliaud et al, 1996). These are the only sites of free cysteinyl residues within the composite chains, but so far there is no direct evidence confirming these linkage positions. We believe that the proteolytic processing of the laminin 5 α3 and γ2 chains is required to facilitate covalent complex formation since all laminin 5 found within these complexes is fully processed. As a result of the covalent association, the valency of the complex for cell binding is doubled (see below), and a binding site for nidogen is contained within γ1. The binding of nidogen allows bridging of the laminin networks with the collagen IV complex and with perlecan and fibulin. It is possible that further stability would result from interactions of the laminin β2, β1 and γ1 VI domains (as occurs with laminin 1 networks, Yurchenco et al, 1992) or with like domains of other laminins within the basement membrane, but this remains unproven and controversial. A second interaction of laminin 5 with the basement membrane results from the strong interaction of the N-terminal end of laminin 5 with the N-terminal (Parente et al, 1991; Li et al, 1993) NC-1 domains of collagen VII (Rousselle and et al, 1997). This interaction does not involve the α3 chain, but instead utilizes either the β3 chain short arm or the residual three laminin EFG-like repeats of the fully processed γ2 chain. Both the interaction of the laminin 5-6/7 complex with the lamina densa components,

and the laminin 5 - collagen VII NC-1 interaction are likely to occur, as the ratio of monomeric to complexed laminin 5 is about 1:1 in both skin and amnion (Champliaud et al, 1996).

The major cell binding site of laminin 5 is located near the C-terminal G domain of the α3 chain. The G domain is also proteolytically processed *in vitro* and *in vivo*, with loss of the 4th and 5th G repeats. The epitope of antibody BM-165 that inhibits cell attachment (Rousselle et al, 1991) is contained with the first G repeat (Champliaud and Burgeson, unpublished finding), therefore the binding site of integrin α6β4 is in the vicinity of the G domain. As laminins 6 and 7 also contain the α3 chain (Champliaud et al, 1996), they can, and do (Rousselle, Amano, and Burgeson, unpublished results) promote epithelial cell attachment as well as laminin 5. Therefore, the laminin 5-6/7 complex contains two cell binding sites per molecule.

In order for the anchoring filaments to stabilize the attachment of the epithelium to the papillary dermis, they must interact with both elements. The NC-1 domain of the anchoring fibril protein collagen VII is embedded within the lamina densa and the anchoring plaques. We have previously shown that the NC-1 domain can bind collagen IV (Burgeson, 1986), but more recently we have demonstrated a direct and stronger binding to monomeric laminin 5 (Rousselle et al, 1997). From the ultrastructure of the anchoring fibrils, it is obvious that the mechanism of stromal matrix binding involves entrapment of dermal fibrous elements. In order for this entrapment to occur, collagen VII must be present as antiparallel dimers. Several years ago, we proposed the participation of the collagen VII NC-2 domain in this process (Bachinger et al, 1990; Lunstrum et al, 1986). We suggest that the NC-2 domain of one molecule recognizes and binds a specific site on the helical domain of an adjacent molecule. This initiates an 60nm overlap of the C-terminal ends of the molecules, allows the NC-2 domain of the second molecule to bind the helix of the first molecule, and brings the overlapped triple-helices and their contained unpaired cysteinyl residues into register, resulting in disulfide exchange and covalent stabilization of the dimers (Morris et al, 1986; Lunstrum et al, 1987). After covalent stabilization, the NC-2 domains are proteolytically excised. The removal of the NC-2 domains has been documented *in vivo*, and DEB cases in which mutations alter the structure of NC-2, or in which NC-2 fails to be cleaved have been identified (Bruckner-Tuderman, 1995). Despite an absence of formal proof, this model has prevailed for a number of years. Binding interactions of anchoring fibrils with other stromal components may also occur, but have not yet been observed.

Bis-2-chloroethyl sulfide is a bifunctional alkylating agent that induces cutaneous deepithelization and slows wound healing (Chauhan et al, 1993; Chauhan et al, 1995). In

addition to being cytotoxic to keratinocytes (Vindevoghel et al, 1994), sulfur mustard causes separation of the epithelium from the underlying basement membrane. The blister plane is immediately below the epithelial basolateral surface within the lamina lucida. Recent studies show that the blister floor contains the lamina densa components collagen IV, and the EBA antigen (collagen VII), while the roof contains the hemidesmosomal proteins BPAG1 and 2 (collagen XVII) (Monteiro-Riviere and Inman, 1995). Laminin (probably the $\beta 1$ and $\gamma 1$ chains were detected), fibronectin, and GB3 (laminin 5) were primarily found on the blister floor, but were also seen associated with the detached epithelial surface. The phenotype observed is strikingly similar to that observed in patients with JEB. The distribution of basement membrane epitopes described by Monteiro- Riviere and Inman (1995) is consistent with a loss of function of the laminin 5 G domain and/or integrin $\alpha 6\beta 4$. The occasional presence of laminin 5 at the blister roof is consistent with its crosslinking to integrin $\alpha 6\beta 4$, or a loss of junction of the N-terminus of laminin 5 and/or collagen VII NC-1 domain.

The ability of sulfur mustard to crosslink laminins has been documented, and the alkylation of EHS laminin diminishes the ability of keratinocytes to attach to it (Zhang et al, 1995). However, the physiological significance of these findings is unclear since EHS laminin is not a natural epithelial attachment substrate, and keratinocytes bind to it poorly relative to their adhesion to laminin 5. What the studies do clearly indicate is that (1) basement membrane molecules can be alkylated by sulfur mustard, and (2) alkylation disrupts their recognition by keratinocyte receptors (presumably integrins).

Direct support for the involvement of laminin in the vesication response was argued by Lindsay and Rice (1995) where they report that laminin extracted from the skin of sulfur mustard treated mini-pigs was specifically and partially degraded. While the data are somewhat confusing relative to what we now know about the epithelial laminins, it is likely that what was detected was not degraded laminin 1, but instead intact laminin 5 - the subunit chains of which are truncated relative to laminin 1. The reported relative molecular weights are consistent with this possibility. These authors report an absence of crosslinking due to the sulfur mustard treatment, suggesting that derivatization alone may be sufficient for blistering to occur.

The molecular basis of epithelial migration over denuded basement membranes remains unclear. The general theory that integrins mediate the process is very likely correct. However, the integrins involved in migration are not necessarily those involved in stable adhesion of mature epithelia. During wound healing, it seems that $\alpha 6\beta 4$ integrin, a key hemidesmosomal

participant in stable adhesion, is downregulated and is replaced by addition integrins. The integrins expressed by the migratory cell largely reflect the composition of the substrate they encounter. For example, in wounds that penetrate the basement membrane are covered by a provisional matrix of fibrin, fibronectin, various collagens, and tenascin (Clark et al, 1982; Mackie et al, 1988). Under these conditions, the fibronectin receptor integrin $\alpha 5\beta 1$ is prominent on the keratinocyte surface (Grinnell, 1992). When keratinocytes migrate onto a denuded basement membrane, integrin $\alpha 6\beta 4$ is likewise downregulated, and hemidesmosomes are endocytosed. Integrin $\alpha 3\beta 1$ - which has broad substrate binding specificity, including laminins - is rapidly upregulated. As migration proceeds, the keratinocytes produce copious amounts of laminin 5, from the leading migratory keratinocyte to the original epithelial boundary. Other laminins, collagen IV and collagen VII are not deposited by these migratory cells, therefore basement membrane assembly lags behind epithelial coverage. Instead, the neoepithelium migrates upon a laminin 5-rich substrate. Several cells away from the migratory front, keratinocytes begin to reassemble hemidesmosomes, but only after epithelial coverage does new basement membrane synthesis begin. Basement membrane assembly appears to begin at hemidesmosomes, and spreads laterally. If the basement membrane is sufficiently intact to be recognized by the reforming hemidesmosomes, they initially form over clusters of anchoring fibrils (Gipson et al, 1983). If the basement membrane is occluded by cell debris, a new basement membrane is assembled over the debris, and the neobasement membrane and the old membrane are ultimately remodeled.

Based upon the literature evidence (cited above) that sulfur mustard alkylates basement membrane components, and that alkylation disrupts their recognition by integrins, we speculate that the loss of rapid healing of sulfur mustard induced blisters is due to structural modification of the basement membrane. Our own preliminary data suggests that providing laminin 5 - the natural keratinocyte adhesion substrate - stimulates migration and neobasement membrane assembly. We propose to test the hypothesis that providing exogenous human laminin 5 to the blister floor and continuously to the forming epithelium will substitute for the damaged basement membrane, promoting wound coverage and basement membrane assembly.

Recent studies related to the proposal:

We have successfully employed a number of techniques to assess interactions between proteins that occur within the basement membrane zone.

Type VII collagen dimers. The covalent stabilization of type VII collagen prepared from tissues can readily be detected by routine SDS-PAGE (Lunstrum et al, 1987). However, the type VII procollagen dimers obtained from cell culture supernatant solutions are not disulfide bonded, presumably because the conditions in the medium do not favor disulfide exchange. Therefore, the dimers are more difficult to demonstrate. We have succeeded in visualizing them in the culture supernatant medium by rotary shadowing (Lunstrum et al, 1987). We believe that NC-2 is required for initiation of dimer formation but this has not been directly tested.

Laminin 5 - type VII collagen. We have recently shown the direct binding of laminin 5 to type VII collagen by solid phase binding assays. A variety of ECM substrates were applied to the substrate, and soluble laminin 5 (A) or the type VII collagen NC-1 domain were separately added at varying concentrations. Of the tested materials, laminin 5 and type VII collagen interact most strongly. The covalent adduction of laminin 6 to laminin 5 that occurs at the N-terminus of laminin 5 significantly reduces the binding signal, suggesting that the short arms of laminin 5 (or their processing remnants) participate in the interaction. Laminin 6 alone does not bind type VII collagen, therefore, the α3 chain is not involved in the interaction. Since the short arm of the γ2 chain is nearly completely removed by processing, the most likely domain binding type VII collagen is within the β3 short arm. We have previously shown that the type VII collagen NC-1 domain binds collagen IV (Burgeson et al, 1986), and this is confirmed in these studies, but the binding is less avid than seen with laminin 5. This assay is extremely useful provided the reagents are of sufficient purity and if multiple controls are provided. Measurements of the binding of laminin 5 to types VII and IV collagens is presently being performed using a Biacore reactor within the facilities of Michael van der Rest in Lyon. These studies have recently been published (Rousselle et al, 1997).

Laminin 5. We have recently shown that laminin 5 binds heparan by heparan sepharose chromatography. The 4th and 5th repeats of the G domain of α3 are required for binding and presumably contain the binding site. Domain G5 contains the heparan binding consensus sequence KKLRIKSK, but the activity of this sequence has not been directly demonstrated.

The covalent adduction of laminin 5 to laminins 6 or 7 has recently been published (Champliaud et al, 1996). There we speculate that the adduction is mediated by a disulfide bond between the laminin 5 β3VI domain and the laminin 6/7 α3III domain, based upon the presence in these locations of unpaired cysteinyl residues. This model predicts that laminin 5 should bind laminin 6, and the expressed domain β3VI will inhibit the binding. This can also be tested by solid phase assays. The binding of laminin 5 α3 chain to epithelial cells (Rousselle et al, 1991; Carter et al, 1991) through integrin α6β4 (Niessen et al, 1994) has been reported

and confirmed now by many laboratories. Our antibody BM-165 prevents the binding, and recognizes an epitope in the α3 G1 domain, but the cell binding site within the α3G domain has yet to be elucidated.

Can purified ECM proteins or their subdomains facilitate epithelial adhesion during normal wound healing? or sulfur mustard induced blistering? We have only recently begun to explore the possible use of exogenously applied laminin 5 to promote wound closure and basement membrane assembly. The results are very encouraging. *In vitro,* we find that laminin 5 applied to keratinocyte substrates dramatically increases keratinocyte, but not fibroblast, migration over the regions to which laminin 5 was applied. The migration rate of keratinocytes over laminin 5 coated plastic surfaces is 4.5 fold greater than over surfaces coated with BSA, collagen I, or laminin 1. We have also applied laminin 5 to fetal bovine skin that had been deepithelized. There too, keratinocytes rapidly repopulated any surface that had been exposed to laminin 5. In preliminary studies, we had destroyed the mesenchymal cells in the skin by repeated freezing and thawing. The procedure was not optimal and has since been abandoned since it caused ruptures within the dermis probably due to ice crystal formation. However, in the laminin 5 treated samples, keratinocytes rapidly replaced not only the epidermis and the hair follicles, but also cells migrated into the dermal ruptures and formed large intradermal cysts. Similar appearing cysts occurred with the untreated skin samples, but the migration of the keratinocytes, particularly into the dermal ruptures was markedly slower, and not readily evident after ten days of culture.

We have also observed the effects of exogenously added laminin 5 upon the rate of basement membrane formation *in vitro*. We have had considerable experience with the dermal equivalent model using keratinocytes grown upon the surface of fibroblast contracted collagen gels (Nishiyama et al, 1994). We have consistently seen that while keratinocytes and mesenchymal cells rapidly localize basement membrane components to the basolateral epithelial surface, basement membrane assembly is extremely slow or does not occur at all. Assembly can be accelerated by using first trimester fetal keratinocytes, thereby allowing visualization of a nearly normal basement membrane by transmission electron microscopy after two weeks of culture. Only a wispy and fragmented structure was observed after the same time when neonatal keratinocytes were used. This observation implied that the fetal cells were simply more biosynthetically active, and thereby some unknown critical concentration of basement membrane components was more quickly reached. We therefore attempted to increase the rate of assembly by adding laminin 5. Initially we simply coated the collagen gel surface with laminin 5 prior to the addition of keratinocytes, assuming that providing a continuous supply of laminin 5 to the epithelial surface would be of little utility

since it was unlikely to penetrate the intact neoepithelium. The results were disappointing; no basement membrane was visible at 14 days. Despite my pessimism, Dr. Nishiyama in the lab added soluble laminin 5 (100μg/ml) to the cell culture supernatant. To my great surprise, a robust, although not entirely continuous basement membrane was visible in the laminin 5 treated dermal equivalents after only two weeks. Further, when we repeated the study using bovine keratinocytes and soluble human laminin 5, we saw that the newly formed basement membrane contained human laminin 5, suggesting that in the submerged cultures, the keratinocytes were capable of transporting laminin 5 from the solution to their basal surface. This observation led Dr. Amano in this lab to evaluate the active transport of human laminin 5 from the upper chamber of transwell cultured bovine keratinocytes to the lower chamber. Quantitation of the human laminin 5 in the lower chamber by sandwich ELISA indicated that only a small quantity of laminin 5 was actively transported. However, when Dr. Amano examined the transported molecules, he found that the fraction of laminin 5 molecule which contained an unprocessed (200kDa) α3 chain were selectively translocated to the lower chamber. He subsequently showed that the unprocessed laminin 5 molecules could be specifically selected by heparin sepharose chromatography. Since processing from 200kDa to 165kDa occurs by cleavage of domains α3 G4 and 5, we hypothesize that these contain a heparan binding site similar to other laminins. Examination of the predicted amino acid sequence of α3 indicates a possible heparin binding motif in domain G5. These observations suggest that a heparan sulfate containing molecule participates in the active transport of laminin 5 by epithelial cells. We are currently attempting to block transport using blocking antibodies to the syndecans and to perlecan. Syndecan is the most likely candidate, since we have seen colocalization of syndecan and laminin 5 in adhesion complexes of primary cultured keratinocytes.

The potential importance of these experiments is that they suggest that unstratified epithelial cells can concentrate exogenous laminin 5 at the basement membrane zone. This may be used to advantage to facilitate both wound closure, basement membrane assembly, and epithelial stability. Consistent with this hypothesis is the observation by Vito Quaranta (Quaranta, 1996) that exogenous rat laminin 5 stabilizes the attachment of the corneal epithelium to the corneal stroma during storage of corneas for transplant, and the rat laminin 5 is likewise incorporated into the human basement membrane. This may be a normal physiological mechanism for stabilization of developing epithelia since we have observed high concentrations of laminin 5 in amniotic fluid samples (Marinkovich et al, 1994), suggesting that its presence in the fluids bathing the epithelial surfaces of both the fetus and the amniotic membranes may be necessary for the development of basement membranes in these rapidly

growing structures. These encouraging studies also suggest that exogenously added laminin 5 may promote the stability of non-keratinized mucosal epithelium. For example, it may prove useful if applied in solution to the eyes of individuals with cicitricial pemphigoid (Kirtschig et al, 1996).

Complementary to these studies, we have begun a collaboration with Dr. Shioya in Japan to evaluate the use of laminin 5 to increase the success of the transplantation of keratinocyte sheets to large wounds. Dr. Shioya heads the leading Japanese group developing and using keratinocyte transplantation to burn victims. His group has extensive experience with mouse models of the protocols. Preliminary animal studies are extremely encouraging. The application of laminin 5 to debrided full thickness wounds on nude mice increased the "take" of the transplants from sporadic and highly variable to nearly 100% successful transplantation. While the number of studies performed to date are too small to be definitive, together with Dr. Shioya's group we are extremely anxious to improve the procedures and prepare for human trials. The experience gained in these animal studies may demonstrate a general utility of laminin 5 in wound healing. It is entirely possible that laminin 5 may promote reepithelization of blisters of some EB patients and rapid closure may increase the stability of the newly formed basement membrane. This can be evaluated using the transplantation of patient keratinocyte sheets or patient-cell-populated dermal equivalents to nude mice.

Hypothesis

This proposal is based upon the hypothesis that the epithelial-basement membrane instability caused by bis-2-chloroethyl sulfide alkylation is due to: derivatization of the G domain of the laminin 5 $\alpha 3$ chain which disrupts the structure of that domain essential to its recognition by hemidesmosomal integrin $\alpha 6\beta 4$; and/or derivatization of the laminin 5 N-terminal domain essential to binding to collagen VII NC-1 domain; and/or derivatization of integrin $\alpha 6\beta 4$; and/or derivatization of collagen VII NC-1 domain. Failure of bis-2-chloroethyl sulfide alkylation induced blisters to heal efficiently is due to the presence of derivatized or crosslinked basement membrane components crosslinked into the alkylated basement membrane which are not well recognized by integrins on the basolateral surface of keratinocytes repopulating the basement membrane. We hypothesize that normal healing would be restored if the derivatized basement membrane were removed, but considerable scarring would result. Alternatively, we will attempt to restore the normal keratinocyte migratory substrate - laminin 5 - to the deepithelialized basement membrane to promote rapid wound coverage. We

speculate that restoration of coverage will stimulate the normal destruction and replacement of the damaged basement membrane. It is of direct interest that the recent publication of Smith et al, (1997) provide additional evidence in support of this hypothesis. Of several tested basement membrane components, only laminin 5 showed a progressive decrease with loss of expression during the time period of sulfur mustard vesiculation in a pig model.

We have developed the technology to produce human laminin 5 in quantities sufficient for the below proposed studies, and have considerable experience with both *in vitro* and *in vivo* models that recapitulate basement membrane assembly.

Body of Report

Methods

Methods related to goal 1:

Isolation of primary human keratinocytes.

Keratinocytes were obtained from human neonatal foreskin as described by Marinkovich et al, (1993). Briefly, Human foreskins were washed in PBS and then in 90% ethanol, and then incubated in PBS containing fungizone, penicillin and streptomycin at 4°C for 30 min. Foreskins were cut into 5mm x 5mm squares, and incubated with dispase overnight at 4°C. The epidermis was pulled from the dermis with forceps, and was then incubated in trypsin-EDTA at 37°C for 30 min. The enzyme activity was neutralized by the addition of fetal bovine serum. Cells were washed with Gibco serum free keratinocyte culture medium and cultured until 70% confluent. Cells were detached with trypsin-EDTA and frozen in liquid N2 (1-2 x 10^6 cells/vial).

Keratinocyte culture for laminin 5 production.

Keratinocytes were grown in Gibco SF keratinocyte growth medium. Primary keratinocytes were expanded in "T75" flasks through 2nd or 3rd passage, and seeded into Nunc Cell Factories (6,320 cm^2)(2 T75 flasks/factory). Normally one expanded vial of primary cells was sufficient to seed each factory. Cells were grown to subconfluency, the medium was changed to DMEM containing 10% FBS, and the media was then changed every two days. Spent medium (1L/factory) was filtered through Whatman No. 1 filter paper, and EDTA (final [5mM]) phenylmethyl sulfonylfluoride (final [50μM]), and N-ethyl maleimide (final 50μM]) were added. The spent medium was frozen at -20°C until 40-70 L were accumulated. Under optimal conditions, the keratinocyte cell factories can be maintained for up to 6 weeks before the cell sheets lift. However, because the factories are easily contaminated, media collection was usually continued for 3-4 weeks, yielding 7 to 10 L of spent medium per factory.

Purification of laminin 5

Modification of our published methods (Marinkovich et al, 1992) were used. Thawed medium (40-70L) was first filtered through a 0.45μm Whatman filter, sodium azide was added to 0.2g/L, and then the medium was passed through a gelatin-sepharose column (305ml gelatin-Sepharose/40L medium). The medium was then circulated (6.1 ml/hr/cm^2) through anti-laminin 5-Sepharose (6F12 [anti-laminin β3 chain]-Sepharose; 4mg 6F12/g CNBr-Sepharose; 200 ml 6F12-Sepharose/column) affinity columns for three cycles. Bound materials were washed with PBS and eluted with 1M acetic acid and immediately neutralized. Optical density at 280nm was monitored and fraction of the peak area were collected and dialyzed 4x versus PBS. Purified materials are immediately treated with diisopropyl fluorophosphate. The sample was then divided into 1ml aliquots and stored frozen at -80°C. The 6F12 affinity column afforded the same degree of purification as the prior use of combined BM-1 and BM-4 [anti-laminin α3 chain] affinity columns, and eliminated the need for a second purification using a BM140 [anti-laminin β3 chain] column.

Isolation of human collagen VII NC-1

For type VII procollagen purification, proteins secreted from confluent WISH (spontaneously transformed amniotic epithelial cell line) monolayers were collected during a 24 h incubation in serum-free medium containing 100 ng/ml ascorbic acid. Following collection, unattached cells were removed by centrifugation (2,000 rpm, 10 min) and EDTA, N-ethylmalemide and phenylmethanesulfonyl fluoride were added to final concentration of 5 mM, 50 mM and 50 mM, respectively. Spent culture media from WISH cells were passed sequentially over 25 ml of gelatin-Sepharose (Pharmacia Fine Chemicals, Pharmacia Biotech, France) and 100 ml (120mg IgG/column) of NP32 (mAb anti-NC-1)- Sepharose (Pharmacia Fine Chemicals). Affinity chromatography on gelatin Sepharose was necessary and sufficient to remove fibronectin from the conditioned media as checked by immunoblotting with a polyclonal antibody against fibronectin. The antigen was eluted with 1M acetic acid, immediately neutralized, and fractions were monitored for absorbency at 280 nm. Pooled fractions were dialyzed against PBS and kept frozen at -20°C. Protein concentration is determined by micro BCA assay (Pierce, Interchim. Monflucon, France).

Extraction of NC1 (type VII collagen)

Collagenase extract of amniotic membrane has been described previously (Bachinger at el., 1990). Affinity chromatography was performed as described above using NP-32-Sepharose. Eluted fractions were analyzed by electrophoresis, pooled and dialyzed against PBS and kept at -20°C.

Collagenase treatment of Type VII collagen

Incubations with bacterial collagenase (CLSPA; Worthington, Freehold, NJ, USA) for 8 h at 37°C in 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, and 5 mM CaCl2 was used to prepare NC-1 from culture media. NC-1 was repurified from collagenase and other digestion products by affinity chromatography as described above. Pepsin digestion (100mg/ml) was for 43 min at 4°C in 0.5 M acetic acid, 0.1 M NaCl.

Assay for keratinocyte attachment to laminin 5.

96 well microtiter ELISA plates were coated with laminin 5 in PBS at 0, 5, 10 and 40 μg/ml, 100μl/well. The plates were incubated at 4°C for 2 hours, and washed extensively with PBS. The plates were then incubated with 1% bovine serum albumin for 30 min. and again washed extensively with PBS. Keratinocyte suspensions were obtained from 3rd or 4th passage cell cultures using trypsin-EDTA and diluted to 1-4 x 10^5 cells/ml with Gibco serum free keratinocyte culture medium. 10μl cells suspension was added to the coated wells of the microtiter plate. The plates were incubated for 30 min at 37°C, and then washed once with cold medium, and then with ethanol at -20°C. Attached cells were fixed with 1% glutaraldehyde in PBS for 10 min, and stained with 0.1% crystal violet in distilled water for 25 min. The wells were washed extensively with water, and the dye was solublized with 2.5% Triton X-100/water (50 μl/well). Color yields were then measured using an ELISA reader at 570nm.

CEES treatment of laminin 5 or type VII collagen NC1 coated wells

CEES was diluted (1 : $10^{2 \text{ to } 6}$) with ethanol and 100μl were added to the wells of laminin 5 or NC1 coated microtiter plates. The plates were sealed and incubated for 2 hr at room temperature. The wells were then washed with ethanol, then with 0.1M Tris-HCl, pH 7.5,

and finally with Gibco serum free keratinocyte culture medium. The medium was removed, and the plates were assayed for keratinocyte adhesion as described above or for solid phase protein-protein binding as previously described (Rousselle et al, 1997).

CEES treatment of laminin 5 or type VII collagen NC1 in solution

Laminin 5 was dissolved in PBS to 200 µg/ml, and NC1 was dissolved in PBS plus 0.2M NaCl to 200 µg.ml. To each 100 µl aliquot, CEES (1:100 in ethanol) was added to final dilutions of $1:10^{2\text{ to }6}$ with rapid mixing at room temperature. The aliquot tubes were sealed and incubated for 2 hours at room temperature. Following incubation, Tris-HCl was added to 0.1 M, pH 7.5, and the samples were desalted on Sephadex G50 (2 ml bed volume) eluted with 0.1 M Tris-HCl, pH 7.5, 0.2 M NaCl. The samples were evaluated by SDS-PAGE.

Methods related to goal 2:

Full thickness skin grafts

Two recipient graft beds in anesthetized mice were ethanol and povidone iodine sterilized and 1cm diameter bilateral full thickness excisions were performed down to the panniculus carnosus. Graft beds were kept moist with Ringer's saline. Human grafts (obtained from surgery performed for unrelated causes, usually circumcision or breast reduction) were placed on the mouse wounds, covered with an occlusive dressing, then with a Band Aid, and held firmly with a compressive dressing which was wrapped around the circumference of the mouse thorax, and stapled on the ventral side of the mouse. Several turns of adhesive tape were applied to further secure the bandage against the efforts of the mouse to remove it. The grafts are inspected after 7 days and have taken after 2 months.

Keratinocyte sheet grafts:

Isolation of primary keratinocytes

Normal human keratinocytes were isolated from neonatal foreskin as described by Marinkovich, 1993. Second and third passage foreskin keratinocytes were grown in a 3:1 mixture of DMEM and Ham's F12 medium supplemented with 10% FCS, 0.5µg/ml

hydrocortisone (Calbiochem-Novabiochem), 5µg/ml insulin (Sigma) 10^{-10} M cholera toxin (ICN) 10ng/ml epidermal growth factor (Pepro Tech EC) and $1.8x10^{-4}$ M adenin (Sigma). (REF 1: Simon M Green H 1985). The medium was changed every 3 days until the cultures became confluent. No 3T3 J2 feeder cells were used.

Preparation of keratinocyte grafts

As soon as keratinocytes had reached a confluent state, epithelial sheets were washed once with DMEM and detached from the growth substrate by incubation for 45 minutes at 37°C with a solution of Dispase II (0.6 U/ml). The sheets were then washed twice with DMEM (Cellgro^{TM}) and transferred to a circular (1.5cm diameter) adaptic non-adhering gauze (Johnson & Johnson), basal side up and fixed with clips (Biomedical Research Instruments Inc).

Procedure for grafting keratinocyte sheets onto nude mice.

Grafting (illustrated in Appendix figure 1) was performed under sterile conditions in a laminar flow hood. Mice were anesthetized by intraperitoneal injection of tribromoethanol (Avertin) (125mg/kg, Aldrich Chemical Company Inc.). Dispase detached keratinocyte sheets were prepared and held in culture medium on circular mesh transfer grids (Fig. 1A). A circular incision (2cm diameter) was performed through the skin and the panniculus carnosus on the dorsum of the mice and the sheet was deposited with the basal cells facing the muscle fascia (Fig 1B). The gauze was removed from the sheet (Fig. 1 C) and a Fusenig Silicon chamber (Renner GMBH, Dannstadt, Germany) was placed over the graft bed to prevent re-epithelialization by the mouse surrounding skin and fixed with wound clips (Becton Dickinson) (Figure 1D).

Split-thickness grafting of human skin to nude mice.

Congenitally athymic nude mice obtained from a colony maintained at the Mass General Hospital were housed under pathogen-free conditions. At 5 to 7 weeks of age, mice were grafted using normal young adult skin as described by Demarchez and collaborators (Demarchez et al, 1985)

Laminin 5 treatment

Laminin 5 was purified as described above (goal 1). 40µl of a purified laminin 5 solution (final concentration 10µg/ml) or 40µl Ringer's solution were applied every 12 hours following transplant through an opening made in the top of the Fusenig chamber.
At day 2 (48 hours after grafting) mice were sacrificed and graft tissue harvested for histology and transmission electron microscopic analysis.

Light microscopy

Grafts were fixed in 4% formaldehyde and paraffin-embedded. 6µm paraffin embedded sections were stained with hematoxylin and eosin. Grafts showing greater than 10% epithelial-dermal separation along the cross-sectioned-diameter of the grafts were judged not to have taken. Immunofluorescence detection was performed as described by Sakai et al, (1986)

Transmission electron microscopy

Samples were fixed for 2 hours in 1.5%:1.5% glutaraldehyde/paraformaldehyde in 0.1M cacodylate buffer pH 7.4 and 0.1% $CaCl_2$ at 4°C and processed as previously described. (Sakai et al, 1986)

RESULTS

Results related to goal 1:

Using the above procedures, we have produced and characterized the laminin 5 needed for these studies. Following initial purification, the materials were tested for keratinocyte adhesion and evaluated by SDS gel electrophoresis (SDS-PAGE). Preparations that did not promote cell adhesion at a plating density at or below 10 µg/ml were discarded without further characterization. Those preparations shown to promote cell adhesion were evaluated by SDS-PAGE. Electrophoretic profiles typical for active (B), and for inactive (A) laminin 5 preparations are shown in Appendix figure 2. The active preparation contains α3 165kDa, α3 145kDa, β3 140kDa, and γ2 105kDa. This is the usual profile of processing intermediates obtained from cultured keratinocytes (Marinkovich et al, 1992). As judged by SDS-PAGE, the laminin 5 appears to be greater than 95% pure. Laminin 5 is synthesized

as a precursor by keratinocytes (Marinkovich et al, 1992). The profile of the intracellular laminin is α3 200kDa; β3 140kDa; and γ2 155kDa. Immediately upon secretion, α3 is processed from 200 to 165kDa with the loss of domains G4-5. γ2 chain processing is somewhat delayed, with the γ2 chains then migrating with an apparent relative molecular weight of 105kDa, due to cleavage within domain III, removing the majority of the short arm. α3 undergoes a second cleavage to 145kDa, with loss of the short arm. Both in tissue and in cell culture, only about 50% of the molecules undergo this cleavage event. The tissue form of laminin 5 is α3 165/145kDa; β3 140kDa; and γ2 105kDa (Marinkovich et al, 1992). Any cleavage beyond these minimal sizes is considered degradation. As can be seen in figure 2, lane A, this preparation is lacking the 165kDa α3 chain completely. Some amount of 145kDa α3 remains, but the majority of the α3 appears to be fragmented. The cell attachment assays relevant to these preparations is shown in Appendix figure 3. When originally isolated, both of these preparations showed comparable electrophoretic profiles and cell attachment abilities. The preparation shown in figure 2, lane A has been stored for nine months; that in figure 2, lane B has been stored for 3 months (subsequently referred to laminin 5 preparations A and B).

Preparations A and B have been compared using keratinocyte attachment assays (Appendix figure 3, upper panel). Intact laminin 5 present in preparation B showed normal cell binding activity. Significant activity was observed on wells coated with 5-10 μg/ml laminin 5. Preparation A lacking the intact laminin 5 α3 chain showed minimal cell binding at 10 μg/ml, but cell binding could be observed at 40 μg/ml, suggesting that some activity remained in the preparation. This is most likely due to the small amount of α3 145kDa remaining in the preparation. Twelve mg of the preparation shown in figure 2(B) and in figure 3(B) were produced for the studies

Experiments have been performed to test the ability of CEES alkylation to diminish cell binding to laminin 5. As shown in Appendix figure 3 (lower panel), a significant reduction of cell binding was obtained when laminin 5 was derivatized using a $1:10^3$ dilution of CEES (8.5mM). Laminin 5 was derivatized in solution with CEES, and the product was tested for its ability to promote keratinocyte attachment. As shown in Appendix figure 4 (open bars),

cell attachment was significantly reduced (27% of control values) by the 2 hr treatment with CEES diluted $1:10^4$. No attachment was detected with laminin 5 treated with higher CEES concentrations. The derivatized laminin 5 was evaluated by SDS-PAGE. No changes in either the staining intensity or in electrophoretic position were seen with materials treated with CEES at the dilution of $1:10^4$ or at lower concentrations. Laminin 5 treated with CEES at dilutions of $1:10^3$ and $1:10^2$ showed decreased stain intensity, and disperse (both faster and slower) electrophoretic mobilities. It is likely that the materials treated at high CEES concentrations were heavily derivatized causing losses due to altered solubility, and altered mobilities due to changes in SDS binding. However, the laminin 5 treated at the dilution of $1:10^4$ appeared to be recovered with high efficiency, therefore it is reasonable to assume that the decreased cell binding resulted from alkylation.

Two mg of purified type VII collagen NC-1 were produced. Laminin 5 was freshly alkylated with CEES ($1:10^{4\ to\ 6}$) as described above for the cell binding. The newly derivatized laminin 5 showed essentially the same decrease in the degree of cell binding (31% of control values) seen previously (figure 4, hatched bars). Binding to type VII collagen was measured in solid phase as previously described (Rousselle et al, 1997). No significant effects upon binding to type VII collagen were detected either when derivatized laminin 5 was used as the soluble ligand (Table 1 and figure 5) or as the solid phase ligand (not shown) in solid phase binding assays. Further, no effects of alkylation were observed when laminin 5 and collagen VII were coimmunoprecipitated, or when binding was evaluated by rotary shadowing (data not shown). Laminin 5 was then derivatized using CEES at 1: $2.5x10^3$, $5x10^3$, and $7.5x10^3$. Insufficient material was recovered after treatment with CEES at $2.5x10^3$, but enough derivatized laminin 5 was recovered from the other two concentrations to attempt binding to collagen VII. Again, no alterations in binding to collagen VII were seen (data not shown), while cell binding was better than 90% diminished (figure 4, hatched bars). Since treatment of laminin 5 with higher concentrations of CEES resulted in substantial loss of the substrate, higher CEES concentrations were not tried.

CEES alkylation of NC1 resulted in essentially complete insolubility of NC1 using CEES concentrations greater than obtained by $1:10^4$ dilution. The material obtained following

alkylation at concentrations less than the above showed no altered laminin 5 binding by solid phase assays (Table 1 and figure 6).

In an attempt to circumvent the insolubility issue, NC1 was plated onto plastic (8μg/ml) and treated with CEES as described for assays of cell attachment. The wells were then assayed for binding of NC1. No binding was observed in any of the wells other than those containing untreated NC1 which were not treated with ethanol. Ethanol appears to irreversibly eliminate the ability of NC1 to bind laminin 5.

Results related to goal 2: Effects of laminin 5 on normal human skin grafts. Optimization of grafting conditions and control experiments for the effects of laminin 5 upon wounds

Nadia Ouahes and Sandra Baldi attempted to generate animal models for studies of the affects of laminin 5 upon wound healing, and upon keratinocyte transplantation. This work is now essentially complete and is being prepared for publication (Appendix, manuscript 1).

Sandra Baldi has tested the effects of laminin 5 upon the transplantation of human keratinocyte sheets to nude mice. The "take" was measured by histology and histochemistry, and the grafts were evaluated by electron microscopy at 48 hrs. post grafting. The results show clearly that exogenous laminin 5 application enhances the take of the grafts. Keratinocyte grafts to 23 of 26 (88%) treated animals were judged to have taken, while only 10 of 19 (10/19) untreated grafts were judged to have taken. The histology of the grafts showed two interesting and consistent findings. These are illustrated in Appendix figure 7, which shows the histology of the grafts judged to have taken, either laminin 5 treated or untreated. All the laminin 5 treated grafts were more extensively stratified then the untreated (compare fig 7E with 7F), and all the treated grafts showed less cellular necrosis.

The grafts were further evaluated by transmission electron microscopy. Representative results are shown in Appendix figure 8. Low magnification micrographs of the grafts, either laminin 5 treated or untreated, judged to have taken showed greater basal cell necrosis in

the untreated relative to the treated (data not shown). However, the most striking finding was a greater degree of ultrastructurally identifiable basement membrane in the treated grafts relative to the untreated grafts (compare Fig 8 A, B, C, D (untreated) with Fig 8 E, F, G, H (treated)). An ultrastructurally intact basement membrane was observed in the treated, but not in the untreated animals at 2 days post grafting. Therefore, the increased graft take due to laminin 5 treatment appears to be due to an earlier onset of basement membrane assembly, and a lessened degree of cellular necrosis.

In parallel studies not directly related to this contract, but relevant to the biology studied during this contract, keratinocytes from a JEB patient genotyped as laminin β3-/- were similarly grafted to 50 mice and laminin 5 was applied as above. The grafts were evaluated by histology, immunofluorescence microscopy for basement membrane components, and transmission electron microscopy at 7 days. Establishment of the treated grafts occurred in 12 of 25 (48%) cases and in 4 of 25 (16%) untreated cases. In all cases, transmission electron microscopic analyses demonstrated detachment of the neoepithelium upon histological processing and the complete absence of recognizable basement membrane. The applied laminin 5 could not be detected by immunofluorescence in either case following 7 days.

Both studies support the following conclusions: (1) laminin 5 significantly increases the adhesion of epithelial sheets to wound surfaces; (2) the adhesion of grafts is enhanced by exogenous laminin 5 due to an increase in the rate of basement membrane formation; and (3) while laminin 5 increases the initial attachment of laminin 5-/- sheets to the graft bed, continuous laminin 5 production is required to support basement membrane assembly and stability.

Cells from the same patient were transfected with full length β3. Transfectants were selected by their ability to bind to untreated plastic dishes. These cells could be shown to deposit β3 containing laminin 5 upon the culture substrate. The transfected cells were grown to sheets and transplanted to nude mice. The take rate of the transfected cell sheets was significantly higher than the untransfected cells (about one-half of the transfected grafts were judged to have taken, roughly equivalent to the "take rate" of

untreated normal cells. The numbers of mice grafted were too low for the absolute % take to be evaluated with any confidence), however, the grafts necrosed after 2 weeks, probably due to the harshness of the transfection conditions.

Treatment of wounds with laminin 5: effects upon normal wound closure in the absence of CEES treatment.

Nadia Ouahes has studied the effects upon wound healing of the addition of exogenous laminin 5. BM165 is a monoclonal antibody which recognizes the G domain of laminin 5. In vitro studies have found that cell adhesion to laminin 5 was abolished in the presence of BM165 (Rousselle et al, 1991). We undertook an in vivo experiment looking at the effects of laminin 5 and BM165 antibody on wound closure. Human skin transplanted onto nude mice has been shown to be a good model of human wound healing. We performed grafting of adult split thickness skin from mammary reductions onto nude mice. Each animal was grafted on both sides of the dorsum. 1 to 2 months after transplantation, an excisional wound was made in the center of each graft using a 2 mm punch. Daily addition of purified laminin 5 (10 μg/ml) to the wound beds had no effect on epidermal closure (Table 2). In a second set of experiments, BM165 antibody (10 μg/ml) was applied in the wound on one side, and a control antibody (same concentration, same immunological isotype, but irrelevant specificity) was applied on the other side. Wounds were covered with a polyurethane dressing. The wounded areas were then harvested 3, 4, and 5 days postwounding. Of 30 mice analyzed, 20 showed a significant (>20%; the average difference in wound diameter decreased 95% when antibody treated) slowing of the reepithelialization process (Table 3), indicating that blocking the cellular adhesion site of laminin 5 slows keratinocyte migration. Also, hemidesmosomes in these wounds were absent, reduced, or immature (Appendix figure 9).

In the BM-165 treated wounds, the BM-165 antibody was localized within the wound using anti-mouse FITC conjugated IgG. Surprisingly, the antibody was detected only under the portion of the migrating epithelial tongues closest to the initial wound margin (Appendix figure 10, BM-165). BM-165 was not seen under the tip of the leading edge of the tongue. Immunofluorescent detection of laminin 5 in equivalent sections showed that laminin 5 was present beneath the entire epithlelial tongue (Appendix figure 10, laminin 5).

Immunodetection of collagen IV and collagen type VII collagen showed their distribution to match that of BM-165 (Appendix figure 10, collagen IV and collagen VII). Since the BM-165 antibody was present throughout the period of wound closure, it is puzzling why laminin 5 at the tip of the epithelial tongue was not bound by antibody BM-165. One likely possibility is that the laminin 5 and the bound BM-165 antibody at the leading edge of the epithelial tongue are rapidly turning over, such that the resulting BM-165 antibody concentration is below the level of detection. The presence of collagen IV, collagen type VII collagen, laminin 5 and BM-165 together beneath the trailing portion of the epithelial tongue suggests that within that region basement membrane assembly has occurred. It is possible that completed basement membrane assembly reduces the turnover rate of laminin 5.

Discussion

The proposed methodology for the scaled up production of laminin 5 has proven successful. The obtained materials are essentially pure, intact, and show cell binding activity.

Storage of this material (-20 degrees C, in solution) remains a difficulty. Comparisons of materials stored for 9 months with those stored for 3 months show degradation of the $\alpha 3$ chain. It is likely that the globular G1-G3 domains have been degraded, leaving only the long arm domain (which appears to migrate by gel electrophoresis in the position of the $\gamma 2$ chain as would be expected). Since G1 or G2 are likely to contain the integrin $\alpha 6\beta 4$ binding site, the electrophoretic profile is consistent with the loss of cell attachment activity of this preparation. The observation is also consistent with the expectation that the coiled-coil domain is the least sensitive to degradation.

The cell binding activity of laminin 5 is sensitive to CEES alkylation. While the concentration of CEES required to diminish cell binding activity is higher that might be expected, it is similar to the results obtained for cell attachment to laminin 1 alkylated with sulfur mustard (10mg/ml, Monteiro-Riviere et al, 1996). It is likely that the solid phase reaction conditions minimize the sensitivity of laminin 5 to derivatization.

We were also able to demonstrate diminished cell binding to laminin 5 alkylated in solution. Although the effective CEES concentration (dilution 10^4 = 840μM) still seems quite high.

CEES alkylation of laminin 5 appears not to inhibit its ability to bind to type VII collagen. The effect of alkylation of NC1 upon binding to laminin 5 could not be evaluated due to the insolubility of the alkylation product. When the alkylation was attempted using solid phase NC1, even ethanol treatment alone prevented binding to laminin 5.

The keratinocyte sheet transplantation results show a clear increase in the take rate of laminin 5 treated sheet grafts. Histological and transmission electron microscopic evaluation of the grafts from the treated wounds showed an increase in epithelial stratification; an accelerated assembly of epithelial basement membranes; and decreased epithelial necrosis. Of these effects, only the increased graft take rate and increased basement membrane assembly rate were predicted. The observed increases in epithelial stratification and decreased cell death were unexpected. There are no literature reports of effects of laminin 5 upon cell proliferation, and our own experience suggested that cultured keratinocytes doubled at the same rate when cultured with or without the addition of laminin 5 (Amano and Burgeson, unpublished data). Therefore, it is unlikely that laminin 5 has an effect upon cell doubling time. The observed increased epithelial stratification could be accounted for by decreased cell death. In the integrin β4 knockout mouse, Dowling et al (1996) noted increased keratinocyte necrosis they ascribe to apoptosis. These results suggested that the absence of a signal derived from integrin α6β4 (a laminin 5 receptor) might result in apoptosis. In the above described keratinocyte sheet transplantation studies, integrin α6β4 is unaltered by the dispase harvesting procedures (Takeda et al, 1998), however, laminin 5 is no longer detected adherent to the receptor (Takeda et al, 1998). One extrapolation of these data is to postulate that the absence of the integrin α6β4 ligand - laminin 5 - resulting from the liberation of the cell sheet from the culture substrate attenuates a survival signal transduced by α6β4. The result might be apoptosis of a significant portion of the cells of the sheet Further apoptosis would be blocked by the synthesis and secretion of laminin 5. The surviving keratinocytes in the sheet graft would eventually stratify to a full thickness epithelium. Rapid treatment of the detached cell sheet

with soluble laminin 5 results in occupancy of the integrin receptors (Takeda et al, 1998). Therefore, it is reasonable to suspect that the effect of laminin 5 upon epithelial stratification may be due to maintenance of epithelial viability.

We obtained only indirect evidence that laminin 5 stimulates keratinocyte migration **in vivo**. The application of monoclonal antibody BM-165, known to block the interaction between laminin 5 and basal keratinocytes, resulted in slowed wound closure. BM-165 also inhibited hemidesmosomal assembly, resulting in the presence of very few hemidesmosomes, which were disconnected from the keratin intermediate filament network. Laminin 5 has been shown in several studies to promote keratinocyte migration and hemidesmosome assembly in vitro (Zhang and Kramer, 1996). Migration assays in vitro have shown that anti-α3 integrin, anti-laminin 5, and anti-α6 integrin inhibit keratinocyte migration on laminin 5. Incubation of cultured keratinocytes with 804G rat cell line conditioned medium containing laminin 5 results in cell attachment and hemidesmosome assembly (Horima et al, 1995). Purified laminin 5 from this medium added to the culture medium of epithelial cell lines induced rapid cell attachment and spreading (Baker et al, 1996). Also, addition of solutions containing laminin 5 to explanted corneal pieces induces assembly of hemidesmosomes (Baker et al, 1996). In Herlitz' disease characterized by an absence of laminin 5, not only are the anchoring filaments absent, but the hemidesmosomes also appear abnormal. Therefore, interaction between laminin 5 and its $\alpha6\beta4$ receptor on the basal keratinocyte appears to be necessary for hemidesmosomes to assemble. The precise signaling pathways involved remain unclear. Xia and collaborators (1996) recently identified a 80 kD membrane-associated protein termed p80 that is tyrosine phosphorylated following detachment of keratinocytes from laminin 5. Inhibition of p80 dephosphorylation prevented $\alpha6\beta4$-dependent cell anchorage to laminin 5.

Plectin and $\alpha6\beta4$ were present in the rudimentary hemidesmosomes found at the edge of the BM-165 treated wounds (data not shown). According to Sonnenberg (Borradori and Sonnenberg, 1996), they may be the first hemidesmosomal molecules to be assembled in the process of hemidesmosomes formation. In our experiments, we could not see any histologic evidence for epidermal detachment in BM165- treated wounds. The immature hemidesmosomes seen in the treated wounds were associated with slow keratinocyte

migration. Interestingly, BP230 null mice have been reported to have slower keratinocyte migration during wound healing, indicating an essential role of this molecule during reepithelialization (Guo et al, 1995). In those mice, hemidesmosomes were not connected to the keratin filaments. The role of BP180 is less clear, and its ligand is unknown.

The exact effect of BM-165 blockage of keratinocyte attachment to laminin 5 upon the slowed wound closure rates is unclear. It could involve any or all of the above processes. However, the most striking observation derived from these antibody studies (other than delayed closure) was the lack of BM-165 detection at the advancing epithelial margin. This is a very strange finding and suggests that laminin 5 is rapidly turning over prior to basement membrane assembly. If this interpretation is correct, this is an surprising finding. If degradation of the matrix at the migrating epithelial wound tip is required for wound closure, then why would one expect the addition of exogenous laminin 5 to speed closure? Could the delayed closure of sulfur mustard wounds be due to resistance of crosslinked matrix to turnover? Alternatively, the high turnover rate for laminin 5 suggested by these studies may be due to its being bound by antibody. Considerable further experimentation will be required to address these questions.

We have grafted human breast skin to 30 nude mice in preparation for the alkylation-induced blistering studies. As usual, the grafts contract to less than 0.5 cm, which presents no problem for the reported 2mm punch biopsy induced wound studies, as the size of the biopsy can be controlled. However, the blisters induce by alkylation appears less easy to control, and the blister often extends into the mouse epidermis leading to a contamination of the wound with mouse cells. The uniform removal of the blister roof also presents a problem, since the quantitative nature of the work requires a uniform wound diameter. We were unable to solve this problem during the contract period.

Conclusions

From the studies conducted during this project period we can conclude:

1. The proposed methods for production of the requisite amount of laminin 5 and NC-1 are feasible. The laminin 5 preparations vary in composition of processing intermediates, and cannot be indefinitely stored. Nonetheless, sufficient materials with keratinocyte binding activity were produced. Adequate amounts of NC-1 were also been obtained.

2. The keratinocyte attachment activity of laminin 5 in vitro is significantly reduced by CEES alkylation. However, mild alkylation of laminin 5 could not be shown to interfere with of type VII collagen binding It is still possible that alkylation of type VII collagen NC1 could affect binding to laminin 5, but this could not be evaluated due to the insolubility of alkylated NC1.

3. Laminin 5 does appear to work reliably as an epithelial-specific glue in vivo. The increased graft take using laminin 5 is encouraging. the increased graft take appears to be due to two factors: a more rapid establishment of a neo basement membrane; and increased and more rapid stratification of the epithelium. We have indirect evidence that laminin 5 may decrease the apoptotic effects upon keratinocytes removed from their natural substrate. If true, this could account for the increased stratification of the neoepithelium.

4. Keratinocytes migrating upon a wound from the wound edge do use laminin 5 as a substrate. However, normal cells appear to produce sufficient laminin 5 in situ that no increase in migration rate is seen by addition of exogenous laminin 5. While this result was somewhat disappointing, we are still hopeful that exogenous laminin 5 will have a positive effect upon epithelial coverage of the alkylated wound. If alkylation acts effectively like the addition of anti-laminin 5 antibody, then exogenous material may be of benefit.

5. The basement membrane zone underlying migrating keratinocytes is rapidly turned over. This was an unexpected finding, and suggests that turnover of the alkylated basement membrane should be investigated.

The overall results of these studies support the underlying hypothesis, that part of the mechanism underlying epithelial-basement membrane instability induced by bis-2 chloroethyl sulfide alkylation may be due to altered protein-protein interactions.

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Appendix

Figure 1. Illustration of the procedure of keratinocyte sheet grafting to nude mice. Dispase detached keratinocyte sheets were prepared and held in culture medium on circular mesh transfer grids (Fig. 1A). A circular incision (2cm diameter) was performed through the skin and the panniculus carnosus on the dorsum of the mice and the sheet was deposited with the basal cells facing the muscle fascia (Fig 1B). The gauze was removed from the sheet (Fig. 1 C) and a Fusenig Silicon chamber was placed over the graft bed to prevent re-epithelialization by the mouse surrounding skin and fixed with wound (Figure 1D).

Figure 2. Characterization of purified laminin 5 by electrophoretic mobility. Two pooled batch preparations of laminin 5 were compared by 5% SDS-PAGE. Lane A is the profile obtained from a preparation initially made in the Spring of 1997, and stored at -80°C for 9 months before the above analysis. Lane B is the profile obtained from laminin 5 stored under the same conditions for 3 months. The profile seen in lane B is typical for laminin 5 produced by keratinocytes in cell culture (α3 165/145kDa; β3 140kDa; γ2; 105kDa). The materials shown in lane A showed the equivalent profile before storage. The stored preparation has lost the 165kDa α3 chain completely, and the amount of 145kDa α3 is also reduced in relative amount. Two lower Mr bands appear in the preparation shown in lane A, and presumably represent fragments of the A3 chain. The laminin 5 in lane B contains only traces of unidentified bands at approximately 210kDa and 300kDa, indicating the essential purity of the preparation. The gel is visualized using Coomassie Brilliant Blue stain.

Figure 3. Comparisons of the keratinocyte attachment activity of laminin preparations. Upper panel: the cell attachment activity of the laminin preparations shown in figure 2 (A and B) are compared. Lower panel: the laminin preparation shown in figure 2 (B) is compared following alkylation of laminin 5 in solid phase using the indicated CEES concentrations on either 10 or 5 µg/ml laminin 5 coated plates. Values graphed are averages of triplicate determinations and the range of values are indicated by bars.

Figure 4. Comparisons of the keratinocyte attachment activity to CEES alkylated laminin 5. The open and hatched bars indicate different experiments. Inf = infinite dilution (ie. No CEES added). Values graphed are averages of triplicate determinations and the range of values are indicated by bars.

Figure 5. Solid phase binding of laminin 5 and type VII collagen NC1. NC1 was bound to the plate and non-alkylated (L5) laminin 5 or laminin 5 alkylated at the indicated dilutions (10^X = 1: 10^X) was added in solution. Bound laminin 5 was immuno-detected using polyclonal anti-laminin 5, quantitated by ELISA reader, and the results of triplicate analyses were graphed as averages.

Figure 6. Solid phase binding of type VII collagen NC1 and Laminin 5. Laminin 5 was bound to the plate and NC1, or NC1 alkylated at the indicated dilutions (10^X = 1: 10^X) was added in solution. Bound NC1 was immuno-detected using polyclonal anti-NC1, quantitated by ELISA reader, and the results of triplicate analyses were graphed as averages.

Figure 7. Histology of the keratinocyte grafts with and without laminin 5. The panels show representative examples of the "best" (left), "average" (middle) and "worst" (right) cases. Laminin 5 treated grafts are shown in the upper panels and untreated grafts are shown in the lower panels. The sheets to be grafted are shown in the top panels.

Figure 8. Electron micrographs of wounds treated (E, F, G, H) or untreated (A, B, C, D) with laminin 5 at two days post grafting of keratinocyte sheets to nude mice. Little or no ultrastructurally identifiable basement membrane can be seen in the absence of laminin 5 treatment, nor are hemidesmosomes seen. Structures that appear as immature hemidesmosomes at low magnification (B, arrow) do not have the expected appearance at higher magnification (C). Laminin 5 treated wounds show considerably increased basement membrane assembly (E, F, G, H), and the presence of immature hemidesmosomes (G, arrows) even over considerable distances (H).

Figure 9. Electron micrographs of wounds made in grafts of split-thickness human skin to nude mice with and without treatment with BM-165 (anti-laminin 5 α3 chain). In the absence of BM-165, basement membranes and maturing hemidesmosomes are seen (lower left panel). The hemidesmosomes (arrows) show expected connection to the intermediate filament network (asterisk; lower right panel; higher magnification of lower left panel). Treatment of the wounds with BM-165 retards basement membrane assembly (upper left panel), and even in areas where basement membrane can be identified (upper right panel; higher magnification of upper left panel), hemidesmosomes are absent or immature (arrow).

Figure 10. Immunofluorescent detection of BM-165, laminin 5, collagen VII, and collagen IV in BM-165 treated human skin grafts to nude mice. Arrows indicate the leading edge of the epithelial tongue migrating over the wound. BM-165, collagen VII and collagen IV are not continuously visualized to the tip of the advancing tongue, while laminin 5 is present beneath the entire tongue. (E=epidermis; D=dermis). The conjoint presence of laminin 5, collagen VII, and collagen IV indicates that basement membrane assembly has occurred at those sites. The presence of laminin 5 but not BM-165 at the tip of the tongues suggests that the laminin 5 is rapidly turning over in that region.

TABLE 1

Solid phase binding studies of Laminin 5 and Collagen VII

A.

Laminin 5 (solid phase): NC1 soluble, 0-20g/ml

	µg/ml NC1			
0	2	5	10	20
NC1 (nonderivatized)				
0.006 +/-0.008	0.293 +/-0.016	0.765 +/-0.029	0.994 +/-0.012	1.006 +/-0.014
NC1 (1:10^6 CEES*)				
0.009 +/-0.006	0.293 +/-0.010	0.791 +/-0.006	0.980 +/-0.070	1.011 +/-0.005
NC1 (1:10^5 CEES*)				
0.006 +/-0.006	0.299 +/-0.016	0.783 +/-0.019	0.986 +/-0.022	1.030 +/-0.012

B.

NC1 (solid phase): Laminin 5 soluble, 0-20g/ml

	µg/ml LM5			
0	2	5	10	20
LM5 (nonderivatized)				
0.008 +/-0.015	0.317 +/-0.014	0.777 +/-0.027	0.960 +/-0.041	1.009 +/-0.019
LM5 (1:10^6 CEES*)				
0.007 +/-0.010	0.261 +/-0.016	0.694 +/-0.031	0.906 +/-0.068	0.956 +/-0.021
LM5 (1: 10^5 CEES*)				
0.008 +/-0.006	0.261 +/-0.016	0.648 +/-0.012	0.867 +/-0.089	0.989 +/-0.026
LM5 (1:10^4 CEES*)				
0.013 +/-0.014	0.250 +/-0.030	0.779 +/-0.041	0.988 +/-0.049	0.985 +/-0.016

All values are averages of triplicate samples determined by ELISA readings at OD

*indicates the CEES dilution used for the derivatization.

Table 2:
Comparison of wound closure rates of Laminin 5 treated versus untreated wounds

TREATED WOUNDS (Lam 5: 10 µg/ml) Bloc / age of wound / Dmax1 (µm)	CONTROLS (PBS) Bloc / age of wound / Dmax2 (µm)	RESULTS Dmax 1- Dmax2 / Dmax1
L1R 3 d 210	L1L 3 d 160	-31 % SIG
L2R 3 d 200	L2L 3 d 160	-25 % SIG
L3R 3 d 150	L3L 3 d 150	0 % NON SIG
L4R 3 d 80	L4L 3 d 120	33 % SIG
L5R 3 d 115	L5L 3 d 120	4.2 % NON SIG
L6R 3 d 80	L6L 3 d 150	46.7 % SIG
L7R 3 d 115	L7L 3 d 110	- 4.5 % NON SIG
L8R 3 d 100	L8L 3 d 120	16.6 % NON SIG
L9R 3 d 120	L9L 3 d 85	-41 % SIG
AVERAGE		0.03%

Dmax 1 = Maximum diameter measured by H&E stained histological sections of Lm5 treated wounds.

Dmax 2 = Maximum diameter measured by H&E stained histological sections of control, untreated wounds.

% difference = (Dmax2-Dmax1)/Dmax2

Differences in closure rates greater than 20% were considered significant = SIG

Differences in closure rates less than 20% were considered insignificant = NON SIG

Table 3:
Comaprison of wound closure rates of BM-2 treated wounds versus untreated wounds.

1) TREATED WOUNDS (BM-2) Bloc / age of wound / Dmax1 (µm)	2) CONTROLS Bloc / age of wound / Dmax2 (µm)	RESULTS Dmax1-Dmax2/ Dmax1
1 R 3d 140	1 L 3d 160	12.5 % NON SIG
2 R 3d 150	2 L 3d 110	-36 % SIG
3 R 3d 150	3 L 3d 70	-114 % SIG
4 R 5d 50	4 L 5d 0	<-100 % SIG
5 R 5d 40	5 L 5d 30	-33 % SIG
6 L 4d 140	6 R 4d 50	-180 % SIG
7 L 4d 180	7 R 4d 0	<-100 % SIG
8 L 3d 200	8 R 3d 60	-233 % SIG
9 L 4d 120	9 R 4d 40	-200 % SIG
10 L 4d 80	10 R 4d 75	-6.6 % NON SIG
11 L 4d 80	11 R 4d 30	-167 % SIG
12 L 3d 120	12 R 3d 110	-9 % NON SIG
13 L 3d 100	13 R 3d 115	10 % NON SIG
14 L 3d 130	14 R 3d 40	-225 % SIG
15 L 4d 65	15 R 4d 0	<-100 % SIG
16 L 4d 90	16 R 4d 60	-50 % SIG
17 R 3 d 110	17 L 3 d 95	-158 % NON SIG
18 R 3 d 100	18 L 3 d 120	17 % NON SIG
19 R 3 d 210	19 L 3 d 170	-235 % NON SIG
20 R 3 d 125	20 L 3 d 35	-257 % SIG
21 L 4 d 100	21 R 4 d 45	-122 % SIG
22 L 4 d 100	22 R 4 d 30	-233 % SIG

1) TREATED WOUNDS (BM-2l)		2) CONTROLS		RESULTS
Bloc / age of wound / Dmax1 (µm)		**Bloc / age of wound / Dmax2 (µm)**		**Dmax1-Dmax2/ Dmax1**
23 L 4 d	30	23 R 4 d	95	68 % SIG
24 L 4 d	160	24 R 4 d	110	-45 % SIG
25 L 4 d	85	25 R 4 d	100	15 % NON SIG
26 L 4 d	120	26 R 4 d	closed	> -100 % SIG
27 L 4 d	100	27 R 4 d	40	-150 % SIG
28 R 4 d	70	28 L 4 d	40	-70 % SIG
29 R 4 d	95	29 L 4 d	85	-12 % NON SIG
30 R 4 d	100	30 L 4 d	75	-33 % SIG
AVERAGE				-95%

Dmax 1 = Maximum diameter measured by H&E stained histological sections of Lm5, or BM-1 treated wounds.

Dmax 2 = Maximum diameter measured by H&E stained histological sections of control, untreated wounds.

% difference = (Dmax2-Dmax1)/Dmax2

Differences in closure rates greater than 20% were considered significant = SIG

Differences in closure rates less than 20% were considered insignificant = NON SIG

Figure 1.

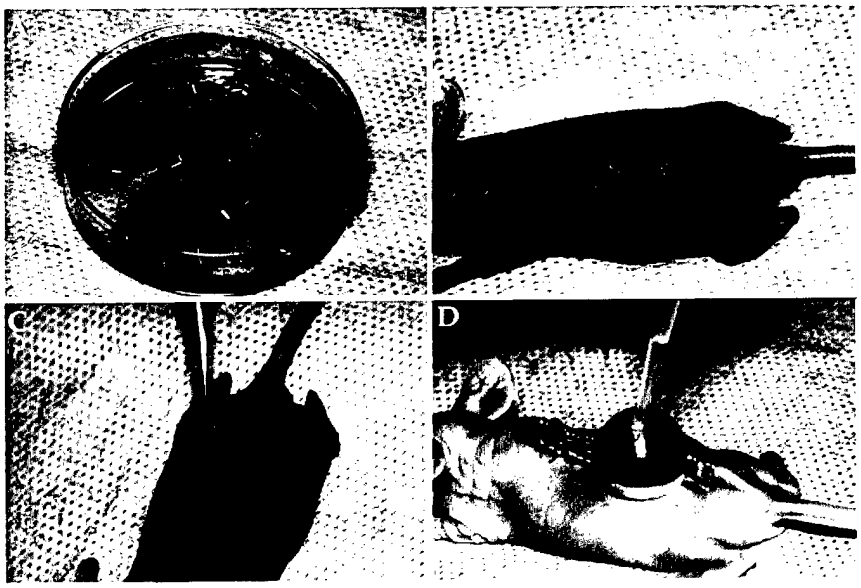


Figure 2.

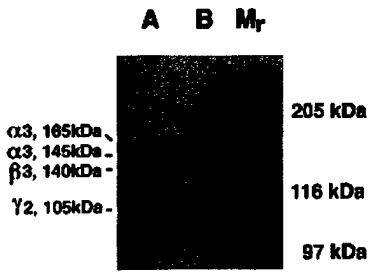

A
B
M_r
α3, 165kDa
α3, 145kDa
β3, 140kDa
γ2, 105kDa
205 kDa
116 kDa
97 kDa

Figure 3

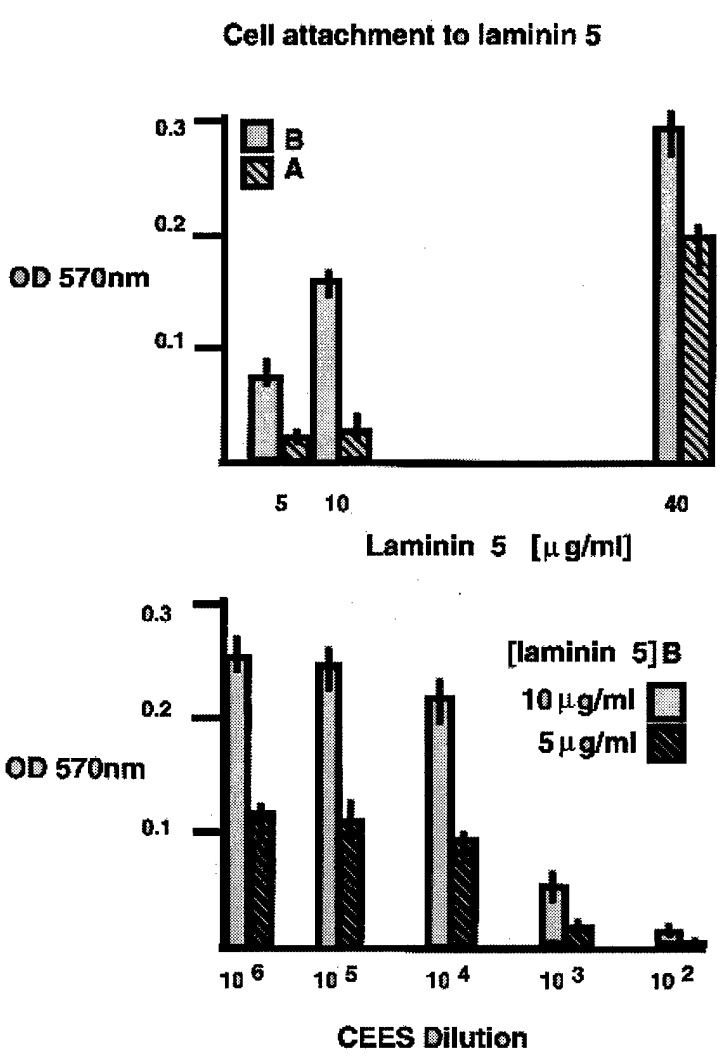

Cell attachment to laminin 5
0.3
0.2
0.1
OD 570nm
B
A
5
10
40
Laminin 5 [μg/ml]
0.3
0.2
0.1
OD 570nm
[laminin 5] B
10 μg/ml
5 μg/ml
10 6
10 5
10 4
10 3
10 2
CEES Dilution

Figure 4

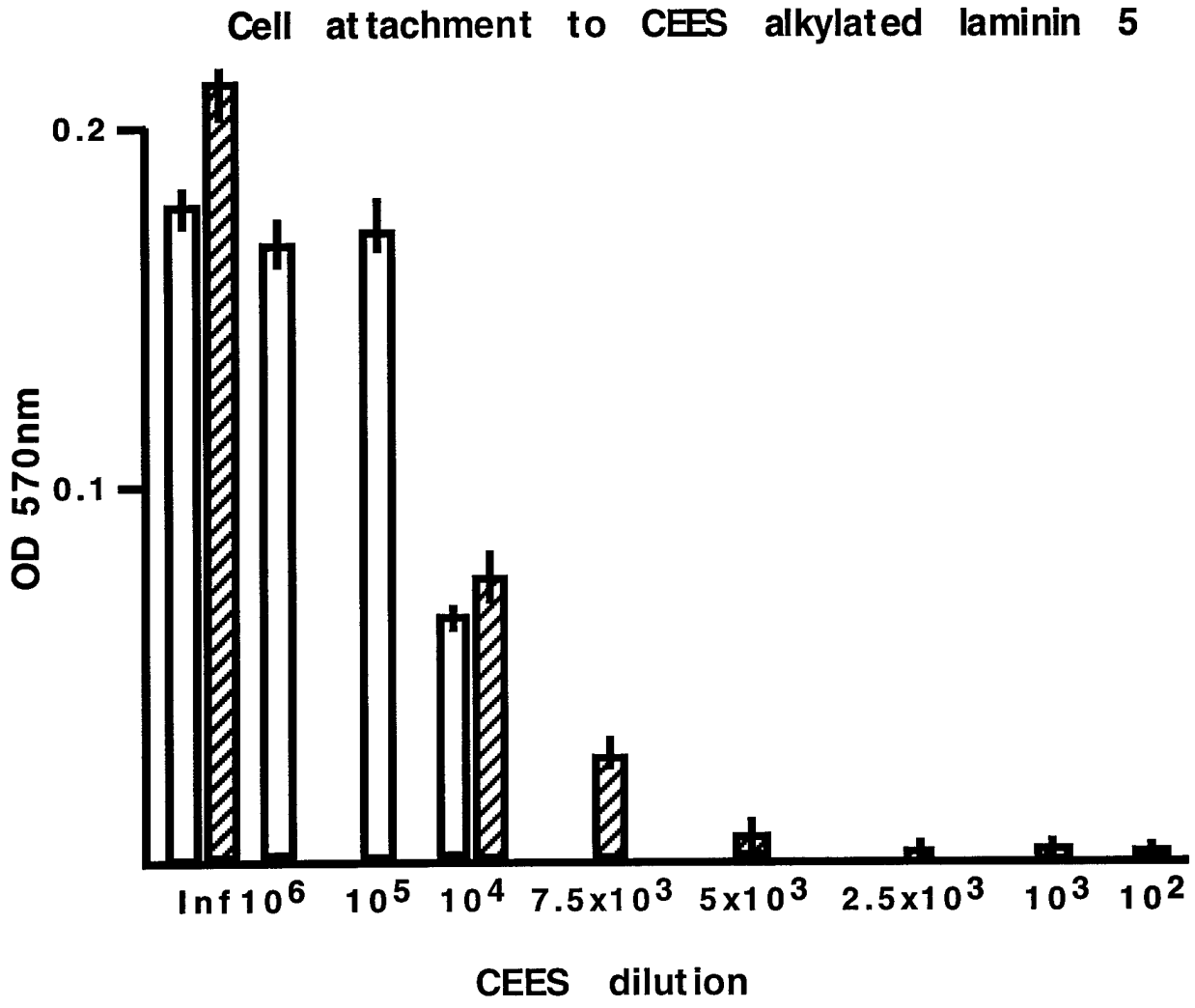

Cell attachment to CEES alkylated laminin 5
0.2
0.1
OD 570nm
Inf
10^6
10^5
10^4
7.5x10^3
5x10^3
2.5x10^3
10^3
10^2
CEES dilution

Figure 5

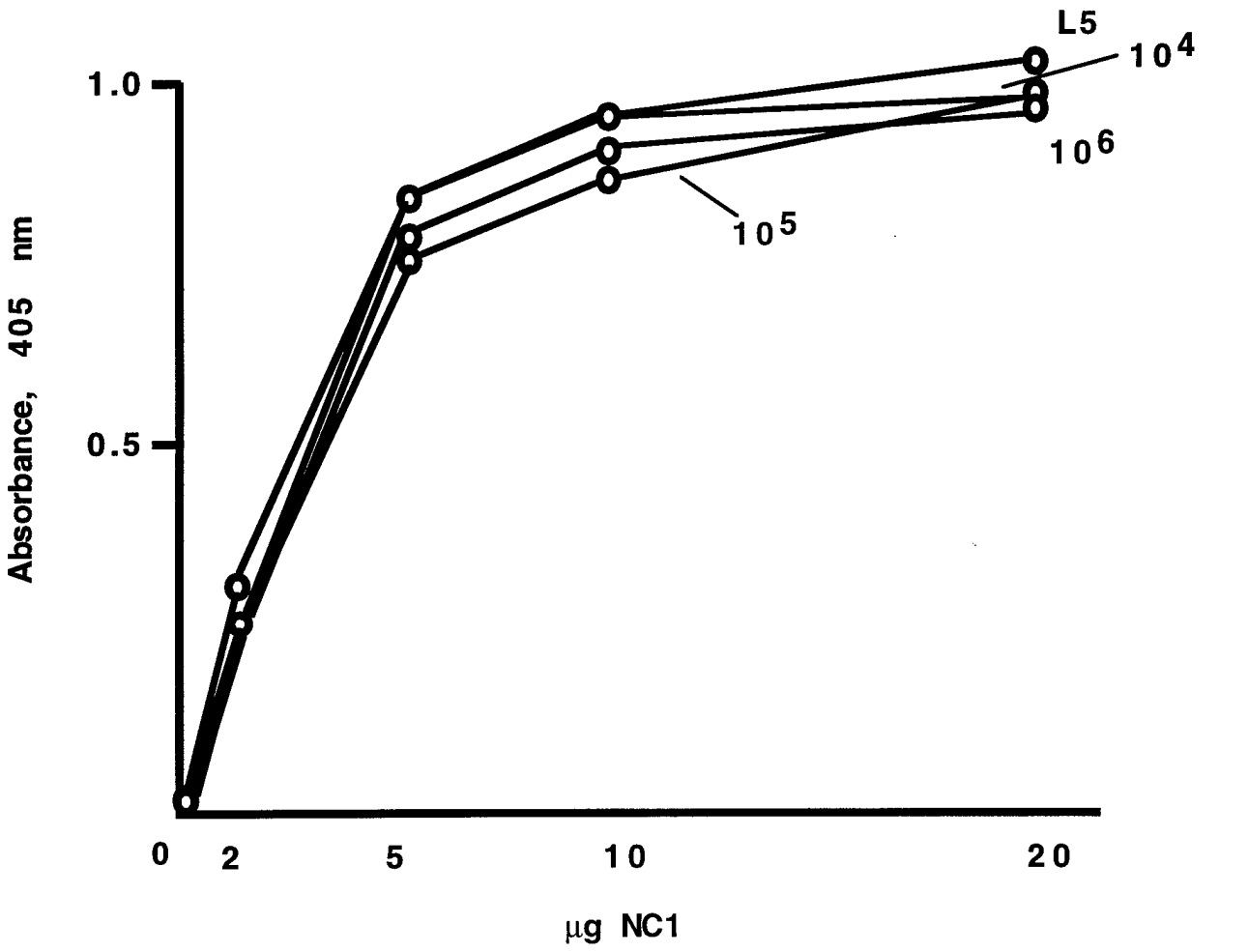

Binding of CEES alkylated Laminin 5 to NC1 (solid phase)
L5
10^4
10^6
10^5
1.0
0.5
Absorbance, 405 nm
0
2
5
10
20
µg NC1

Figure 6

Binding of CEES alkylated soluble NC1 to Laminin 5 (solid phase)

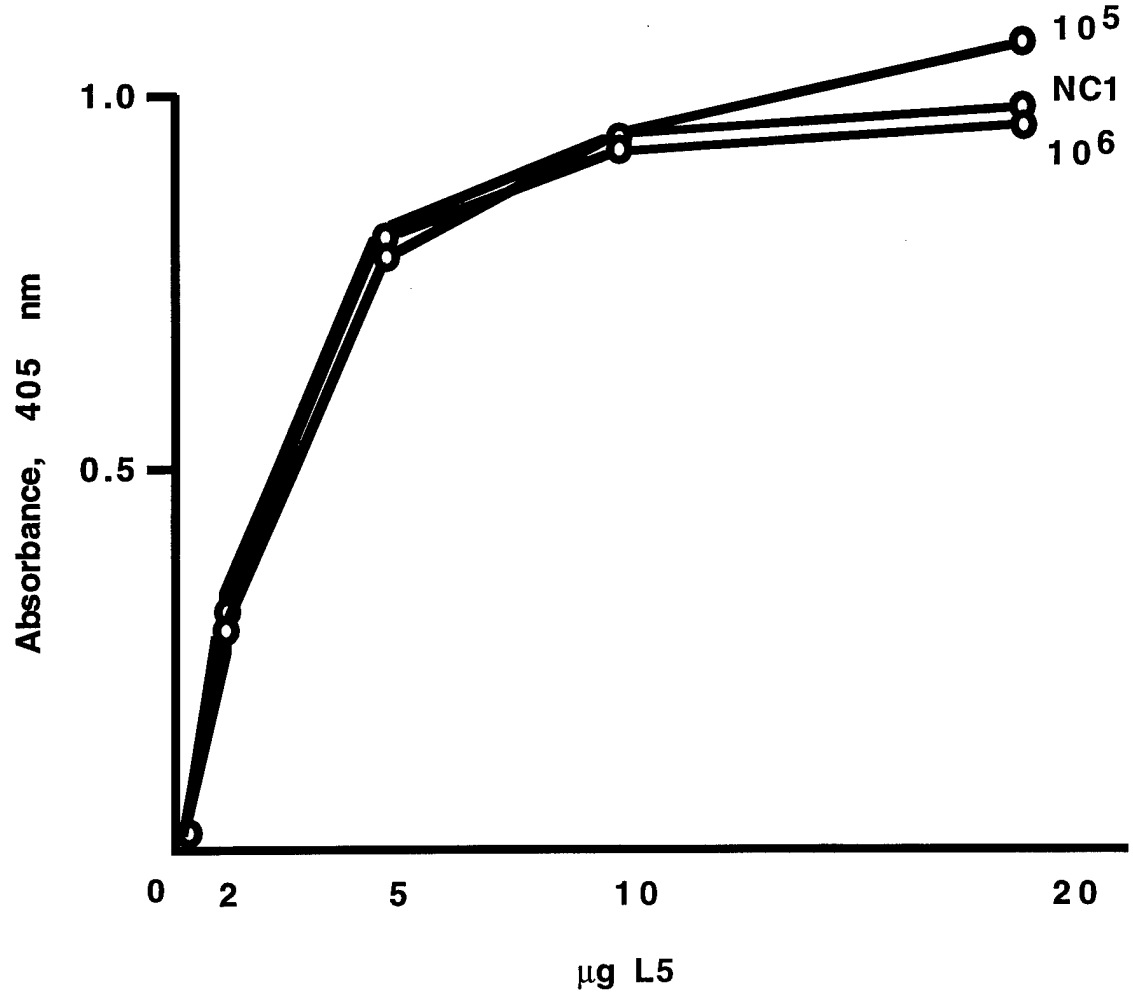


Figure 7.

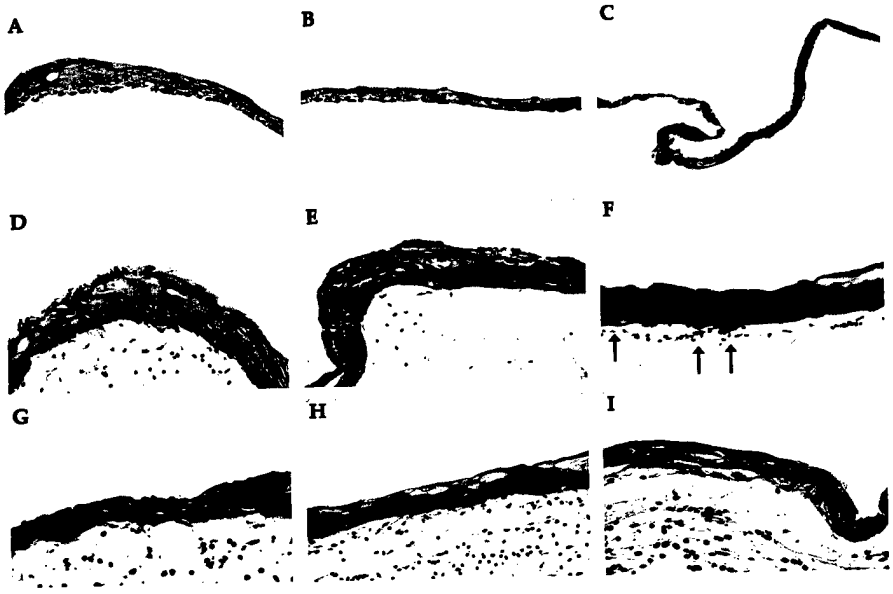


Figure 8.

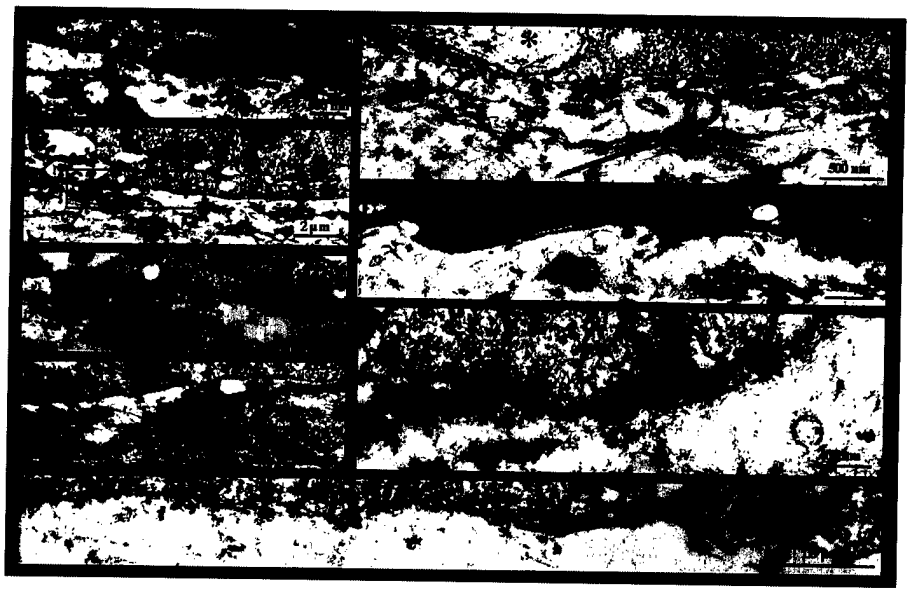
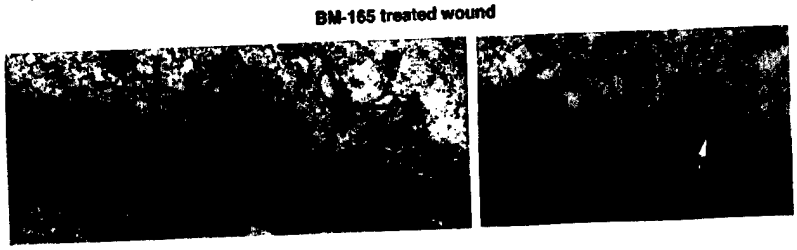


Figure 9.

BM-165 treated wound



Control antibody treated wound

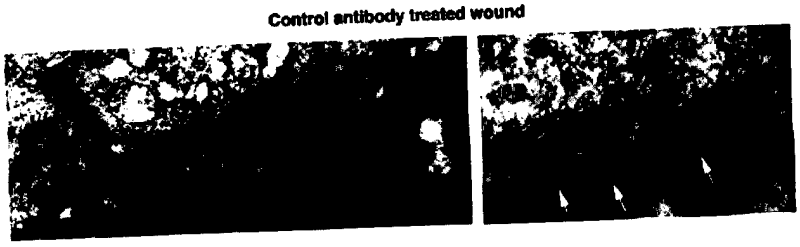


Figure 10.